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U.S. Army Medical Research and Materiel Command ltr., dtd July 23, 2001.

AD_____

CONTRACT NUMBER DAMD17-96-C-6096

TITLE: Reversible Suppression of Menstruction with Antiprogestins

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CONTRACTING ORGANIZATION: Oregon Regional Primate Center
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REPORT DATE: October 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1997	Annual (23 Sep 96 - 22 Sep 97)

4. TITLE AND SUBTITLE
Reversible Suppression of Menstruation with Antiprogestins

5. FUNDING NUMBERS
DAMD17-96-C-6096

6. AUTHOR(S)
Robert M. Brenner, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Oregon Regional Primate Center
Beaverton, Oregon 97006

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

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13. ABSTRACT *(Maximum 200*

The military woman needs a method to delay menses to improve her military readiness. Our goal is to use normally cycling, menstruating nonhuman primates to develop safe, reversible methods of menstrual suppression with late generation antiprogestins. In Year 1, we demonstrated that the Schering antiprogestin, ZK 137 316, administered continuously over 40 days at either 0.05 or 0.1 mg/kg body weight, suppressed overt menses in all animals. Ovulation was inhibited in half the animals by both doses. In some animals that ovulated, minor blood loss, detectable only by vaginal swab, occurred briefly at the end of the cycle. Therefore, chronic antiprogestin treatment inhibited overt menses two ways: either by inhibiting ovulation or by inhibiting endometrial development so that only minimal blood loss occurred at the end of ovulatory cycles. Serum E_2 levels were within normal range in all animals during treatment, and all animals reverted to normal menstrual cycles by the second post-treatment cycle. In summary, we have found a short term, antiprogestin dose that will reversibly inhibit overt menstruation in naturally cycling rhesus monkeys. In Year 2, we will evaluate longer term antiprogestin treatments, examine a new, more potent Schering antiprogestin, and explore how antiprogestins inhibit endometrial development.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Defense Women's Health Research Program antiprogestin, amenorrhea, endometrium, growth factors, menstruation, rhesus macaques	17
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Limited

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Robert M. Brennea Oct. 16, 1997

PI - Signature Date

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INTRODUCTION

The goal of this research is to develop a reversible method of menstrual suppression through use of antiprogestins. These compounds, which include mifepristone (RU 486), onapristone (ZK 98 299) and a new Schering compound, ZK 137 316, are structurally related, synthetic ligands for the progesterone (P) receptor, which antagonize P action through competitive binding at the receptor level. Hodgen's laboratory was the first to report that treatment of cynomolgus monkeys with mifepristone on days 10-12 of the menstrual cycle delayed the mid-cycle LH surge and lengthened the intermenstrual interval from 30 days (control) to 61 days (1). Similar results have been reported for women (2). This suggests that antiprogestin treatment could block ovulation and delay progression of the menstrual cycle. Moreover, long term mifepristone treatment in women with endometriosis blocked ovarian cyclicity and caused amenorrhea (3). Such effects were shown to be dose-dependent, as low dose mifepristone treatment did not affect ovarian cyclicity or menstrual cycle length in women (3,4). Menstrual cycle length was also not affected in bonnet monkeys (5) treated with low doses of onapristone whereas higher doses prolonged menstrual cycle length. Recently (6,7) we presented a complete histological analysis of dose-dependent inhibition of endometrial development in cycling rhesus monkeys treated continuously for 5 months with ZK 137 316. This work also showed that ZK 137 316 could block endometrial development by inhibiting some of the effects of estradiol (E_2) as well as P. We (8) and Hodgen (9) had previously shown that mifepristone could block the ability of E_2 to stimulate endometrial growth. The mechanism underlyng this so called antiproliferative effect of antiprogestins on endometrial growth remains to be established.

The first specific aim of the current contract was to establish a short term antiprogestin regimen with ZK 137 316 that would reversibly block menstruation. Before beginning these studies, we conducted some additional, preliminary dose-finding trials. A convenient endometrial endpoint for ranking of various doses is menses induction in a P-primed endometrium. Although this may seem anachronistic when the ultimate goal is to suppress menses, we have found that doses that induce menses at the end of the menstrual cycle in a P-primed endometrium correlate well with doses that block the ability of both E_2 and P to induce endometrial development during the cycle. Menses induction is a simple, noninvasive assay that requires no surgery. Therefore, we tested the ability of various doses of ZK 137 316 to antagonize P action in rhesus monkeys, and then selected two of these doses for a 40 day menses-suppression trial in cycling monkeys. This report summarizes our progress to date.

METHODS

Animal care throughout these studies was provided by the veterinary staff of the Oregon Regional Primate Research Center (ORPRC). Analysis of serum samples for E_2 and P was performed by the ORPRC Hormone Assay Core (10).

Preliminary dose-finding studies: ZK 137 316 treatment of spayed, hormone treated monkeys

As a first approach to selection of appropriate doses, we tested the ability of ZK 137 316 to block P action and induce menses in ovariectomized monkeys. Artificial cycles were initiated by inserting a 3 cm Silastic capsule (0.34 cm i.d.; 0.64 cm o.d.; Dow Corning, Midland, MI) packed with crystalline E_2 (Steraloids, Inc., Wilton, NH) s.c. into spayed monkeys for 14 days to produce an artificial follicular phase. After 14 days of priming, a 6 cm Silastic capsule containing crystalline P (Steraloids, Inc.) was inserted s.c. for 14 days to stimulate an artificial luteal phase. ZK 137 316 (Schering AG, Berlin) was dissolved in 37.5 % Hanks Balanced Salt Solution, 37% 1,2-propanediol, and 25% ethanol. Beginning on day 14 of the luteal phase, all monkeys were injected daily with ZK 137 316 in vehicle for 7 days. Five doses of ZK 137 316 (mg/kg) were tested: vehicle only (control; n=4), 0.01 mg ZK 137 316 (n=6), 0.03 mg ZK 137 316 (n=6), 0.05 mg ZK 137 316 (n=4), 0.1 (n=6), and 0.15 mg ZK 137 316 (n=3). Vaginal swabs were performed daily for 9 days and the ability of the various doses to induce frank, overt menses (blood detectable on the floor of the cage and on the external genitalia) or minor bleeding detectable only by vaginal swab was recorded.

Effects on menstrual and ovarian cycles of a 40 day antiprogestin treatment with 0.05 and 0.1 mg/kg ZK 137 316 in rhesus monkeys

Untreated, adult cycling rhesus monkeys were monitored for 2 menstrual cycles to document normal cycle lengths for each animal. Beginning on the day after onset of the third menstruation the animals were injected i.m. daily for 40 days with ZK 137 316 dissolved in 37.5 % Hanks Balanced Salt Solution, 37% 1,2-propanediol, and 25% ethanol. Three groups were treated: vehicle only (control; n=4), 0.05 mg/kg (n=6), and 0.1 mg/kg (n=6) body weight. Daily blood samples and vaginal swabs (to detect minor bleeding) were collected during the treatment period until the monkeys demonstrated a full menstruation of longer than 2 days. After menstruating, the monkeys were allowed to rest one menstrual cycle, and then daily blood samples were again collected for one menstrual cycle. All blood samples were analyzed for concentration of E_2 and P. Lengths of the menstrual cycle (or inter-menses interval), length of the recovery period (time to return to menses) and serum hormone levels were compared between treatment groups. Statistical comparison of intermenstrual interval between groups was done by analysis of variance, followed by Fisher's Protected LSD Test (11).

RESULTS

Preliminary study of the effect of various doses of ZK 137 316 on menses induction in artificially cycled monkeys

Table 1 shows a summary of the effects of various doses of ZK 137 316 on menses induction in spayed, artificially cycled monkeys. Serum levels of E_2 and P in these monkeys were 129±26 pg/ml and 4.39±0.48, respectively. ZK 137 316 treatment at 0.01 mg/kg did not induce either menses or vaginal bleeding, 0.03 and 0.05 mg/kg consistently induced vaginal bleeding but not menses, 0.1 mg/kg induced menses in some and bleeding in others, and 0.15 mg/kg induced full menses in all animals. These results were extremely useful as they provided the full range of effects, from no effect at 0.01 mg/kg to full blockade of P action in all animals at 0.15 mg/kg. We selected two of the intermediate doses, 0.05 and 0.1 mg/kg for our first 40 day trial in cycling monkeys, on the grounds that the modest P-antagonistic effects of these doses would be amplified by repeated administration over 40 days.

Short-term suppression of menses with ZK 137 316

Table 2 presents the menstrual cycle lengths of monkeys treated with ZK 137 316 for 40 days. All of the monkeys in the study exhibited normal pretreatment menstrual cycles. Vehicle injected control animals maintained normal length menstrual cycles. However, injection with 0.05 and 0.1 mg/kg ZK 137 316 significantly extended the intermenstrual interval, blocking frank menstruation (menses detected externally) in all of the monkeys during the treatment period (40 days; Table 2). However, in the 0.05 mg group, 4 monkeys showed minor vaginal bleeding, detectable only by swab, on days 27-30 of the treatment period. In the 0.1 mg group 2 monkeys also showed vaginal bleeding detectable only by swab on days 27-28 of the treatment period. ZK-treated monkeys in both groups menstruated between 50 and 63 days after the onset of treatment, and maintained normal menstrual cyclicity thereafter. There was no significant effect of ZK 137 316 treatment on post-treatment cycle lengths (Table 2). Figures 1-3 depict E_2 and P levels in monkeys treated for 40 days with ZK 137 316. During the treatment period, monkeys in the control group expressed normal menstrual cycle patterns of E_2 (Figure 1A) and P (Figure 1B). In the 0.05 mg ZK 137 316 group, 2 of the of the 6 monkeys failed to demonstrate an E_2 surge with E_2 levels rising above 200 pg/ml (compare Figure 2a and c) but non surge E_2 levels were normal (~30-100 pg/ml). In this group, 3 of the 6 monkeys failed to develop a normal luteal phase (compare Figure 2b and d). Similar results were seen in the 0.1 mg group, with 4 of the 6 monkeys not showing an E_2 surge even though non-surge E_2 levels were normal (Figure 3 a and c), and with 3 of the 6 monkeys not developing a normal luteal phase (Figure 3 b and d). In both ZK-treated groups, the monkeys that appeared to achieve ovulation with normal luteal phases maintained elevated P levels for 9-14 days and then showed a P decline to baseline by day 27-30 of the treatment period but failed to

show overt menstruation. This indicates that ZK 137 316 treatment suppressed full, overt menstruation whether or not the luteal phase was normal or blocked. However,1-2 days of bleeding (detectable by vaginal swab only), coincident with the decline in P at the end of the luteal phase, occurred in 5 of the 7 animals that ovulated. Menstrual bleeding was not observed in the ZK 137 316-treated animals that did not develop a luteal phase. During the second post-treatment menstrual cycle (Figure 4), monkeys in all the groups expressed normal patterns of E_2 and P and menstruated normally.

DISCUSSION

We have now conducted a wide range of dose-finding trials with ZK 137 316 in rhesus monkeys and have identified a range of doses from no effect (0.01 mg/kg) to full effect (0.15 mg/kg), as assessed by the menses induction test in spayed, P primed animals. Moreover, we have determined that doses of 0.05 mg/kg and 0.1 mg/kg that partially antagonize P action in the menses induction test will fully block frank menses in intact cycling animals when administered for 40 days. We have further shown that this treatment is fully reversible and that the monkeys regain normal menstrual cyclicity beginning with their first menstruation following treatment.

Suppression of overt menses occurred even in monkeys with normal luteal phases, but some of these animals exhibited brief, minor bleeding, at the time of P decline, which was undetectable except by vaginal swab. Therefore, these doses of antiprogestin either block menses completely or allow what may be called a "silent mense." We have discussed these data with a reseach gynecologist, and she indicates that blood detectable only by vaginal swab would go completely unnoticed by women. Positive vaginal swabs are not the equivalent of so-called spotting or breakthrough bleeding, as the latter implies a loss of more blood with seepage to the external genitalia. We will continue efforts to find a dose regimen that completely blocks all bleeding, but silent menses would certainly be more compatible with military effectiveness than full and frank menstruation. We conclude that short-term antiprogestin treatment may provide a reliable means of reversible menses suppression for military women during deployment.

Treatment with ZK 137 316 at 0.05 and 0.1 mg/kg blocked surge levels of E_2 and attenuated luteal phase P in some of the monkeys. However, all of the animals had E_2 concentrations within the normal non-surge range for rhesus monkeys. This indicates that antiprogestin-treated animals had ample E_2 to maintain the integrity of estrogen target systems (e.g. bone, reproductive tract, heart and circulatory system, vaginal mucosa, etc.) and thus avoid any estrogen-deprivation effects.

Several studies suggest that, in addition to their classical P-inhibiting effects, antiprogestins have 'antiproliferative' or 'cytotoxic' effects on the endometrium. These

are marked by stromal compaction, apoptosis and inhibition of endometrial development (6,12,13), the net effect of which is to block the growth promoting action of E_2 on the endometrium. The cellular mechanism underlying these antiproliferative effects is unknown. However, inhibition of endometrial development is undoubtedly the underlying reason why antiprogestin-treated endometria fail to menstruate normally at the end of an ovulatory cycle.

One of the long-term objectives of this contract is to elucidate the cellular mechanisms responsible for the antiproliferative effects of antiprogestins. Our current working hypothesis is that antiprogestins may induce a mild, sustained myometrial contracture that leads to vascular constriction, reduced endometrial blood flow, endometrial atrophy, and reduced responsiveness to the decline of P in an ovulatory cycle. We plan some experiments to address this hypothesis in the near future.

CONCLUSION

In summary, we have found a short term, dose-regimen with the Schering antiprogestin ZK 137 316 that reversibly inhibits full and frank menstruation in all treated rhesus monkeys. Ovulation was blocked in some but not all monkeys, and in those that ovulated, some, but not all, exhibited a silent menses detectable only by vaginal swab at the end of the ovulatory cycle. Estrogen levels in treated animals were within normal ranges, whether ovulation was blocked or not, which suggests there would be no symptoms of estrogen deprivation. The results mean that a useful technique to suppress menses and enhance the readiness and effectiveness of the military woman can be developed. Future studies will include testing of higher doses, trials of longer term treatments, evaluation of a new, more potent Schering antiprogestin, and an exploration of the mechanism by which antiprogestins inhibit endometrial development.

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Table 1. Effects of various doses of ZK 137 316 on menstruation in artificially cycled monkeys after 14 days of E_2 plus P treatment[1]

	Days Observed								
	1	2	3	4	5	6	7	8	9
0.01 mg ZK 137 316/kg (n=6)	U	U	U	U	U	U	U	U	U
0.03 mg ZK 137 316/kg (n=6)	U	U	U	Bleeding (1)	Bleeding (6)	Bleeding (6)	U	U	U
0.05 mg ZK 137 316/kg (n=3)	U	U	U	Bleeding (3)	Bleeding (3)	Bleeding (3)	Bleeding (3)	U	U
0.1 mg ZK 137 316/kg (n=6)	U	U	U	Bleeding (3)	Bleeding (6)	Bleeding (3) Mense (3)	Bleeding (5) Mense (1)	U	U
0.15 mg ZK 137 316/kg (n=3)	U	U	U	Bleeding (1)	Mense (3)	Mense (3)	Mense (3)	Mense (2)	Bleeding (1)

[1] U indicates menses undetectable. Bleeding indicates blood detectable by vaginal swab only. Mense indicates frank menses detectable externally. Monkeys were injected with ZK 137 316 day 1 through day 7. Numbers in parentheses indicate the number of monkeys within each group showing bleeding or menstruation.

Table 2. Effect of ZK 137 316 treatment for 40 days on intermenstrual interval in cycling rhesus monkeys[1]

	Control	0.05 mg 137 316	0.1 mg ZK 137 316
Pretreatment cycle lengths	28.2±1.0[a]	31.2±1.2[a]	30.1± 1.3[a]
Treatment cycle lengths	26.8±0.88[a]	53.0±5.5[b]	60± 2.2[b]
Posttreatment cycle lengths	27.0±1.8[a]	30.2±5.2[a]	32.1± 2.3[a]

[1] Values represent mean (± SE) menstrual cycle lengths. Means with different superscripts are statistically different ($P<0.01$).

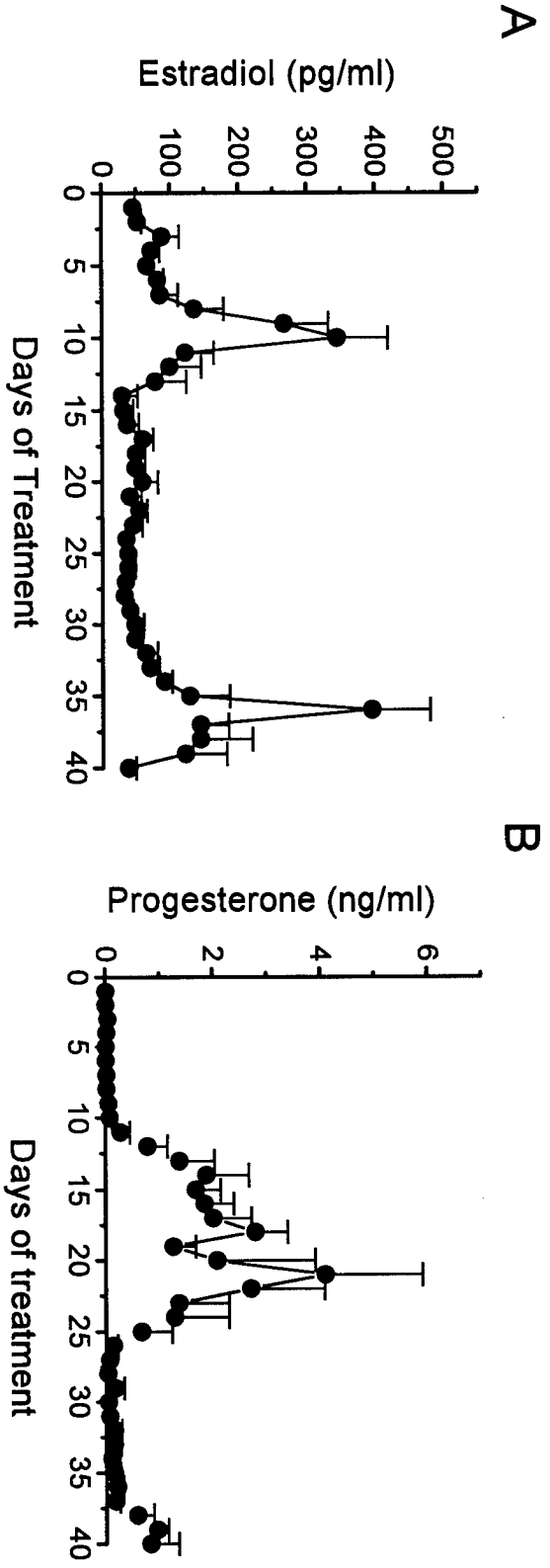
Figure Legends

Figure 1. Mean (±SE) E_2 (A) and P (B) levels in vehicle treated control monkeys (n=4). All the monkeys showed a normal follicular phase E_2 surge and luteal phase rise in P.

Figure 2. Mean (±SE) E_2 and P levels in monkeys treated treated for 40 days with 0.05 mg/kg. A) E_2 levels in monkeys with normal E_2 surges (n=4); B) P levels in monkeys with normal luteal phases (n=3); C) E_2 levels in monkeys that failed to develop an follicular phase E_2 surge (n=2); D) P levels in monkeys that failed to develop a normal luteal phase (n=3).

Figure 3. Mean (±SE) E_2 and P levels in monkeys treated for 40 days with 0.1 mg/kg ZK 137 316. A) E_2 levels in monkeys with normal E_2 surges are shown in (n=2); B) P levels in monkeys with normal luteal phases (n=3). C) E_2 levels in monkeys that failed to develop a follicular phase E_2 surge (n=4); D) P levels in monkeys that failed to develop a normal luteal phase (n=3).

Figure 4. Mean (+SE) E_2 and P levels during post treatment cycles. All the monkeys control (A and B; n=4), 0.05 mg/kg (C and D; n=6), and 0.1 mg/kg groups (E and F; n=6), showed a normal follicular phase E_2 surge and luteal phase rise in P.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300
400
500
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5
10
15
20
25
30
35
40
Days of Treatment
B
Progesterone (ng/ml)
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4
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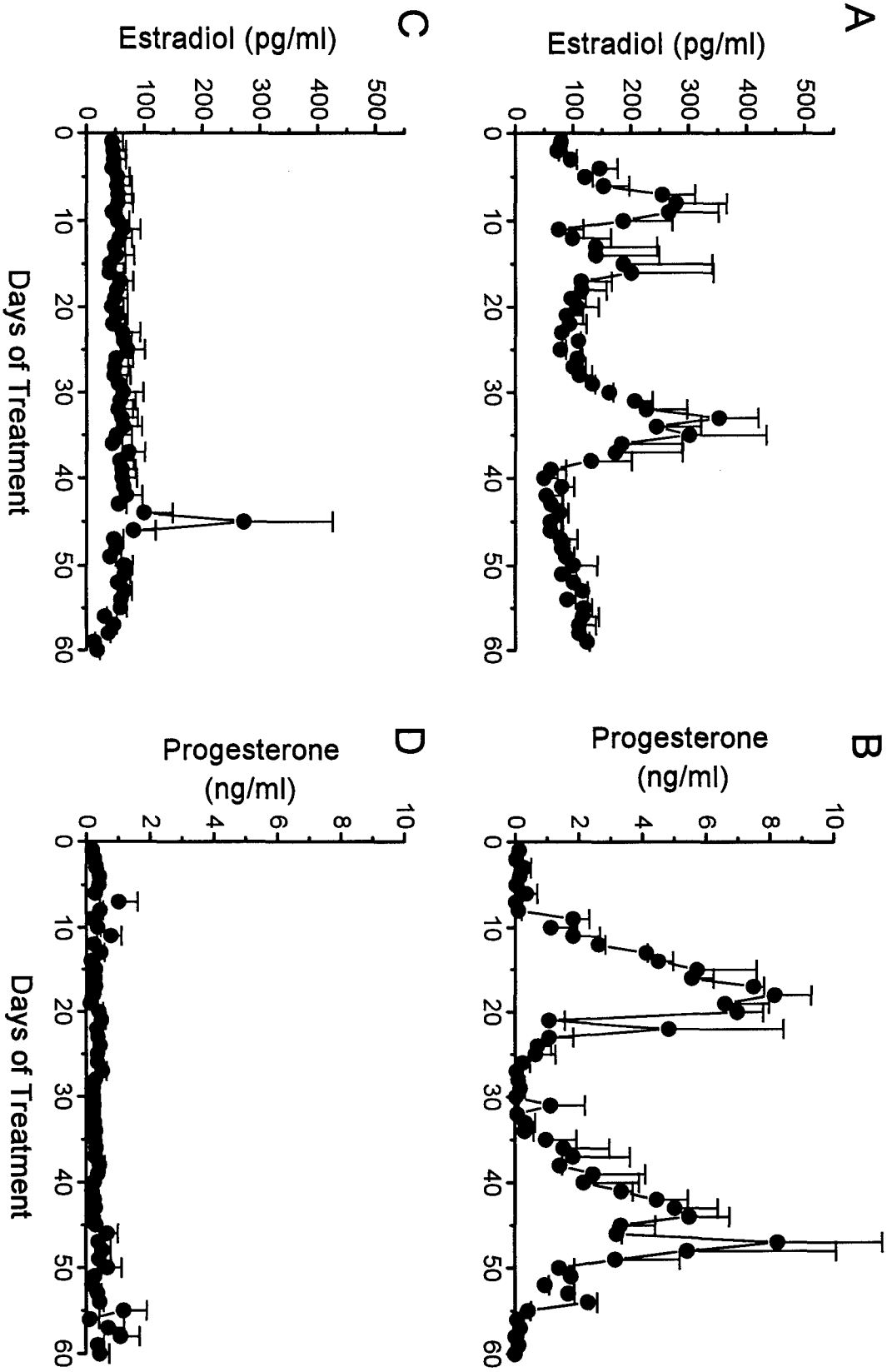
35
40
Days of treatment

Figure 1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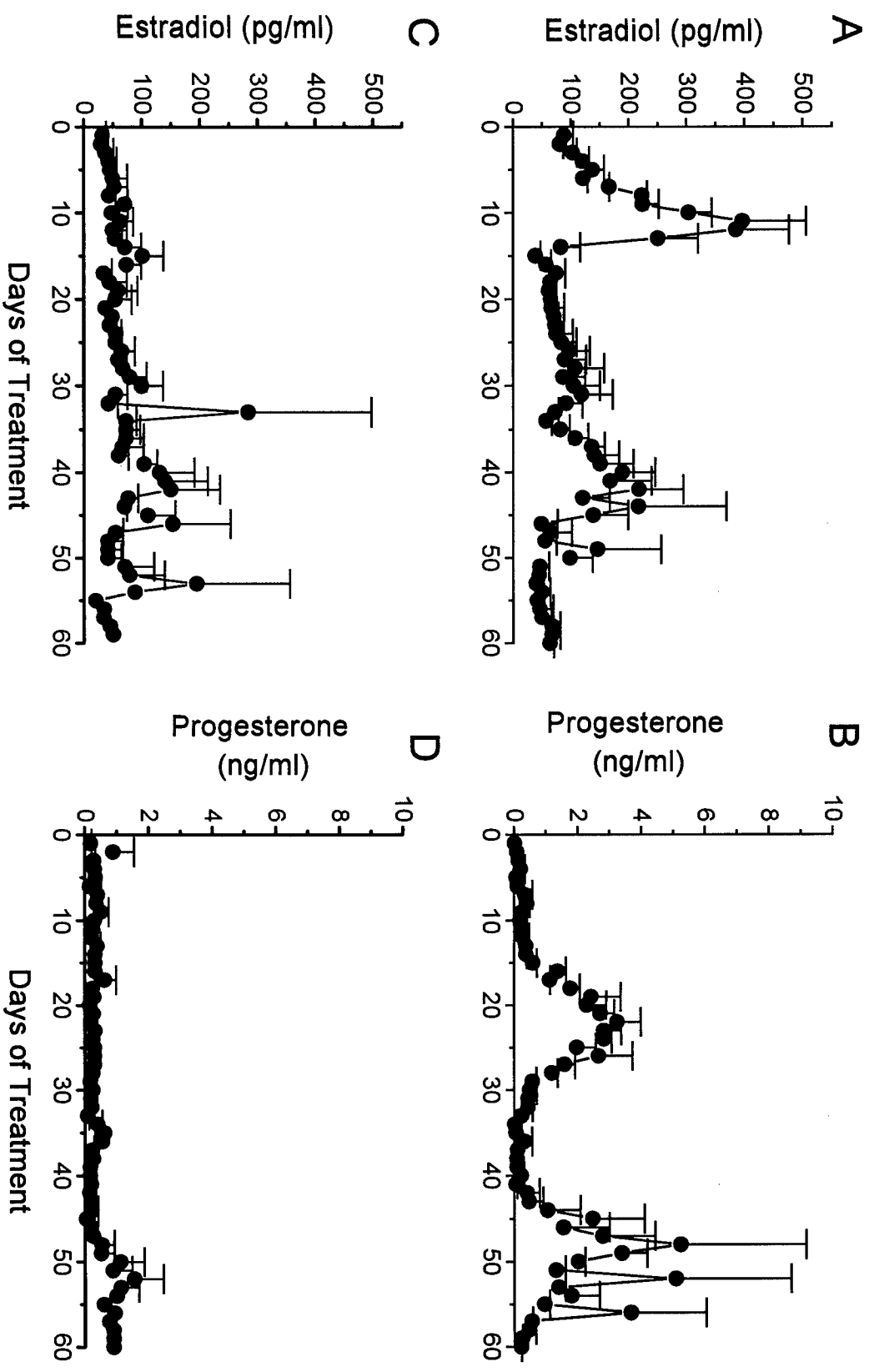
Days of Treatment
Days of Treatment

Figure 2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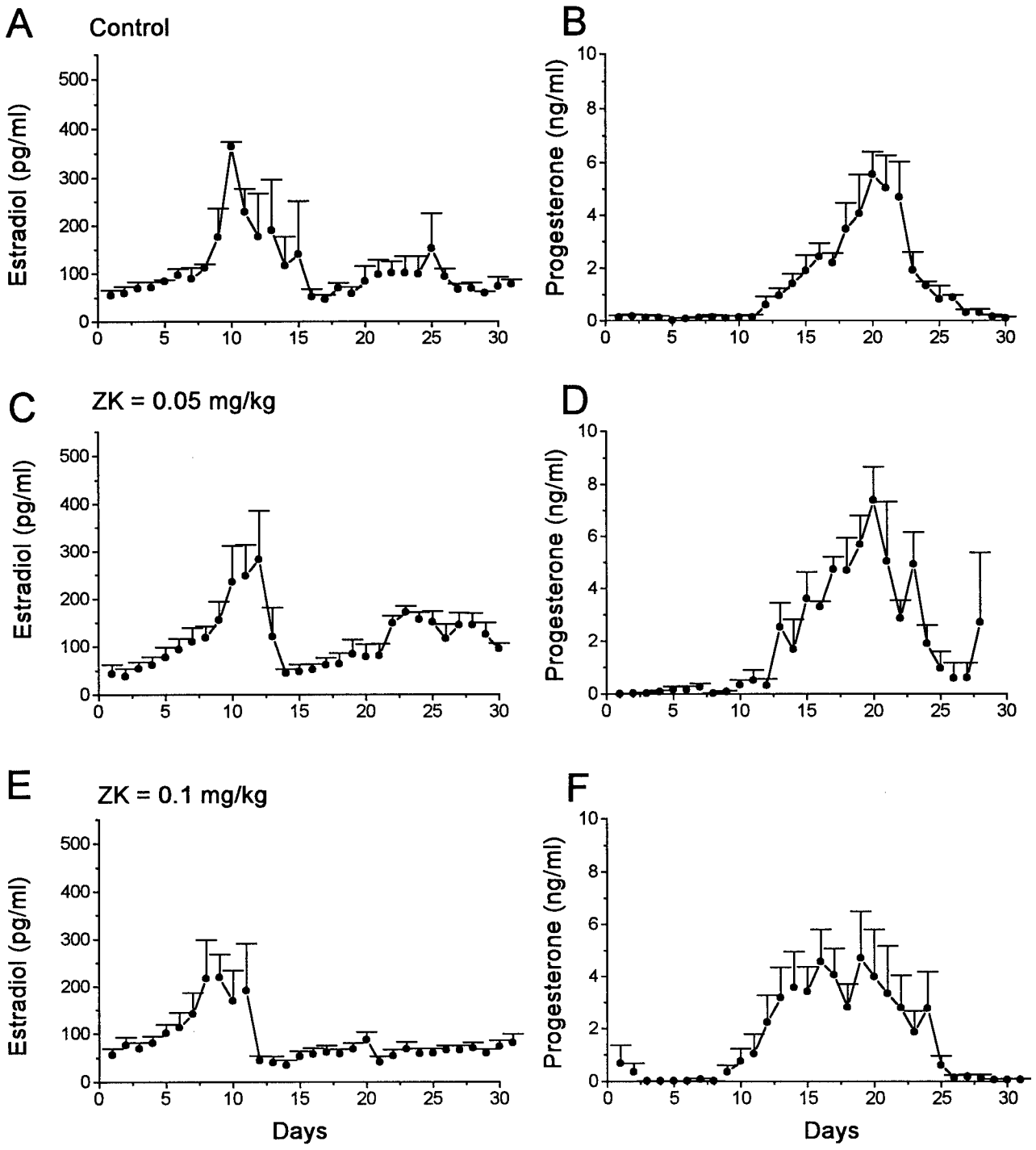
(ng/ml)
Days of Treatment
Days of Treatment

Figure 3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D
Progesterone (ng/ml)
E
ZK = 0.1 mg/kg
Estradiol (pg/ml)
F
Progesterone (ng/ml)
Days
Days

Figure 4

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